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TITLE: Application of Combined Cardioprotective Agents to Preserve Organ Function and Improve Survival during Experimental Hemorrhagic Shock

PRINCIPAL INVESTIGATOR: Robert A. Kloner MD, PhD

CONTRACTING ORGANIZATION: Huntington Medical Research Institutes
Pasadena, CA 91101

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14. ABSTRACT Hemorrhagic shock remains a major cause of mortality and morbidity on the battlefield and in the civilian world after trauma and accidents. Even with restoration of blood volume, organs subjected to hemorrhagic shock can develop ischemia/reperfusion injury and fail. The overall purpose of these studies is to develop novel therapies to improve survival and organ function in the setting of hemorrhagic shock and consider combinations of therapies. So far we have developed a model of experimental shock that simulates blood loss followed by blood return on the battlefield. Early studies suggest that the anesthetic agent isoflurane improved survival and improved recovery of blood pressure. Remote ischemic preconditioning, which can be achieved with inflation and deflation of blood pressure cuffs on the limbs also looks promising and may improve survival, reduces lactate level and improves reticulocyte counts.				
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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Hemorrhagic shock remains a major cause of mortality and morbidity on the battlefield. Even with restoration of blood volume, organs subjected to hemorrhagic shock can develop ischemia/reperfusion injury and fail. This study aims to develop new therapeutic approaches to improve survival and protect vital organs during and after hemorrhagic shock. The therapies with proven cardio-protective properties include: (1) remote ischemic preconditioning, which could be given prophylactically (simple and cost-effective repetitive inflations and deflations of a blood pressure cuff on the arm) to soldiers prior to going into high risk combat situations; (2) the mitochondrial protective agent SS31, which could also be administered prophylactically; and (3) therapeutic hypothermia, which could be produced with a Thermo-Suit device (already FDA approved for hyperthermia) to rapidly cool the body and protect vital organs in case the injury occur. These therapies, alone or in combination, will be investigated to improve overall survival and protect vital organs from ischemia/reperfusion injury of hemorrhagic shock (vs. placebo) in a standardized experimental model of fixed pressure hemorrhage in male and female adult Sprague Dawley rats.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Hemorrhagic shock; remote ischemic preconditioning; mitochondrial protective agent; therapeutic hypothermia; fixed pressure hemorrhage.

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: To test the hypothesis that prophylactic remote ischemic preconditioning improves long term survival and the function and structure of the vital organs (heart, brain, lung, kidney and liver) after experimental hemorrhagic shock, created by withdrawing blood and later reinfusing it, in Sprague Dawley rats of both sexes. **The major tasks** are: (1) from 1 to 6 months, instrument rats and perform studies for Specific Aim 1 (n = ~90 rats); (2) from 7 to 8 months, collect and collate data; (3) in 9th month, analyze data to assess survival, organ function and structure and mitochondrial function. **Milestone(s) Achieved** – Determine whether remote ischemic preconditioning improves long term survival and improves the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. This study is about 80% completed.

Specific Aim 2: To test the hypothesis that prophylactic administration of mitochondrial protective agent, SS31, improves long term survival and the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. **The major tasks** are: (1) from 10 to 15 months, instrument rats and perform studies for Specific Aim 2 (n = ~90 rats); (2) from 16 to 17 months, collect and collate data; (3) in 18th month, analyze data to assess survival, organ function and structure and mitochondrial function. **Milestone(s) Achieved** – Determine whether prophylactic administration of mitochondrial protective agent, SS31, improves long term survival and improves the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. Study to start in year 2.

Specific Aim 3: To test the hypothesis that moderate hypothermia administered in the setting of hemorrhagic shock will improve long term survival and preserve the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. **The major tasks** are: (1) from 19 to 24 months, instrument rats and perform studies for Specific Aim 3 (n = ~90 rats); (2) from 25 to 26 months, collect and collate data; (3) in 27th month, analyze data to assess survival, organ function and structure and mitochondrial function. **Milestone(s) Achieved** – Determine whether moderate hypothermia administered in the setting of hemorrhagic shock will improve long term survival and preserve the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. Study to start in year 2-3.

Specific Aim 4: To test the hypothesis that a combination of promising therapies tested in aims 1, 2, and/or 3, which have been shown to have a positive effect, will have additive (or synergistic) effects on survival and organ preservation in the setting of hemorrhagic shock. **The major tasks** are: (1) from 28 to 33 months, instrument rats and perform studies for Specific Aim 4 (n = ~90 rats); (2) from 34 to 35 months, collect and collate data; (3) in 36th month, analyze data to assess survival, organ function and structure and mitochondrial function. **Milestone(s) Achieved** – Determine whether a combination of promising therapies tested in aims 1, 2, and 3 have additive (or synergistic) effects on survival and organ preservation in the setting of hemorrhagic shock. Study to start in year 3.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

This annual report covered 1 Sep 2016 – 30 Aug 2017, and focused on the major goals listed in the Specific Aim 1.

From 1 Sep 2016 to 30 Oct 2016, we ordered a blood gas analyzer from NOVA biomedical, set up an account in IDEXX for blood chemical analysis, and ordered rats for the study of Specific Aim 1.

From 1 Nov 2016 to 17 Jan 2017, we started to instrument rats and perform studies for Specific Aim 1. Fixed-pressure hemorrhagic shock was induced in Sprague-Dawley rats (mixed gender). The rats were anesthetized with intraperitoneal ketamine and xylazine (90mg/kg and 10mg/kg), and were heparinized with 500 U/kg heparin. Hemorrhagic shock was induced by removing blood via the femoral artery catheter to attain a mean blood pressure of 30 mm Hg. Mean blood pressure was maintained at 30 mm Hg for 60 minutes and then the collected blood was returned over the next 30 minutes. At 180 minutes after closely monitoring the animals and returning the shed blood, the catheters were removed from the blood vessels and the rats were allowed to recover from anesthesia. We chose these parameters based upon pilot studies at the time the grant was submitted. However, in these pilot studies recovery was only acute, up to 3 hours and we did not know whether rats would survive long time after this protocol. We performed the studies on 15 rats (8 rats in preconditioning group and 7 rats in control group), and observed that only 1 rat in the female preconditioning group fully recovered from anesthesia and survived to 6 weeks; while the rest of the rats died within 24 hours after the hemorrhagic shock procedure. This first set of data suggested that the goals of approximately 33% or more rats surviving over 3 days in the control group could not be reached in rats that were subjected to a mean blood pressure of 30 mm Hg for 60 minutes. Therefore we needed to find the right hemorrhagic shock blood pressure level and duration, considered shortening the recovery time from anesthesia, and explored another anesthetic regimen. Our goal was a dose finding study that would result in about a 33% or more survival but not a 75% to 100% survival, so that we could test the efficacy of our different therapies on survival. We requested the review and approval process for the modification to our protocol, and it was approved on 19 Jan 2017.

From 6 Feb 2016 to 15 March 2017, in order to compare the effects of a different anesthesia regimen on the survival rate after hemorrhagic shock, Sprague Dawley rats (both genders) were anesthetized with either intraperitoneal ketamine and xylazine (K/X, 90 mg/kg and 10 mg/kg; n=6), or with isoflurane (5% isoflurane induction and 2% maintenance in room air; n=6). Rats were intubated and ventilated with room air, heparinized, and hemorrhagic shock induced by withdrawing blood from the left carotid artery to a fixed mean blood pressure of 30 mm Hg for 60 minutes, followed by 30 min of resuscitation with shed blood. Rats remained anesthetized for one hour during which hemodynamics were monitored (femoral artery catheter); and then allowed to survive for 6 weeks. The survival rate at 6 weeks in rats receiving K/X in this study was 0/6 (0%); the survival rate in rats receiving isoflurane was 5/6 (83%; p<0.05 by Fisher's exact test) (Figure 1 A). All deaths occurred within 24 hours of the shock study; rats surviving past 24 hours survived for 6 weeks. Recovery of blood pressure during the resuscitation phase was significantly improved in the isoflurane group compared to the K/X group (Figure 1 B, C and D). Rats that receiving isoflurane had normal values of blood gas. Rats that receiving K/X showed early significantly elevated potassium and chloride levels, the PH and base excess levels were significantly lower compared to the isoflurane group. At 6 weeks, all rats in the isoflurane group were neurologically intact, and tetrazolium chloride staining showed no evidence of myocardial, brain or kidney infarction. A subjective histopathology score based on H&E staining was performed to grade the injury level of brain, heart, lung, kidney and liver. H&E stained lung sections showed patchy lung

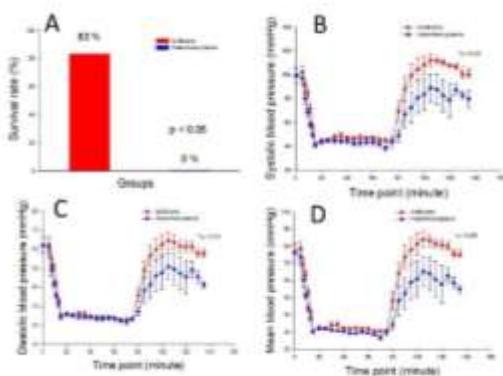


Figure 1: Comparison of survival rate and blood pressure between isoflurane and ketamine/xylazine (K/X) group. Panel A: The survival rate in rats receiving isoflurane was significantly higher than that receiving K/X. Panel B, C and D: Recovery of blood pressure during the resuscitation phase was significantly improved in the isoflurane group compared to the K/X group. (B) Systolic BP; (C) Diastolic BP; and (D) Mean BP. Red=isoflurane, Blue=K/X

lesions, including patchy areas of consolidation, lost alveoli morphology, erythrocyte leaking into alveoli and lymphocytes near bronchi. There were no architectural changes, inflammation, fibrosis and necrosis in H&E stained brain, heart, kidney and liver sections. These results suggest that isoflurane may have protective effects in the setting of hemorrhagic shock, marked by improved recovery of blood pressure early after transfusion/ resuscitation, as well as long term survival without end-organ infarction. Because the protective effects of isoflurane were so striking they could mask the effects of prophylactic remote ischemic preconditioning; therefore we decided to find the critical duration of 30 mmHg under anesthesia with intraperitoneal ketamine and xylazine for the goals of approximately 33% or more rats surviving over 3 days in control group. The findings with isoflurane were unexpected but may be some of the most important findings to date. If it is protective then this may have important clinical implications. We would like to pursue isoflurane in the future and consider adding it to ischemic preconditioning if that therapy works.

From 22 Mar 2016 to 5 Jun 2017, in order to develop a reliable experimental hemorrhagic model for developing new therapeutic strategies for preventing hemorrhagic-shock-induced death, we determined how long a duration of hypotension (30 mmHg) could be tolerated before death was inevitable; that is a dose finding study in which survival went out to 6 weeks (rather than 3 hours as in our initial pilot study for this grant proposal). Sprague Dawley rats (mixed gender) were anesthetized with intraperitoneal ketamine and xylazine (90mg/kg and 10mg/kg), intubated and ventilated with room air. Rats were heparinized and subjected to blood withdrawal via catheter implanted in the left carotid artery in order to establish hypotension at a level of 30 mmHg. Rats were subjected to this level of hypotension for 30, 35, 45, or 60 minutes and then the shed blood was reinfused within 30 min (n=6 in each group). The rats were monitored under anesthesia for one hour and then allowed to survive for 6 weeks. Rats exposed to 30 minutes of 30 mmHg had a 6 week survival rate of 4/6 (66.7%); rats subjected to 35 minutes of 30 mmHg had a 6 week survival rate of 1/6 (16.7%); rats subjected to 45 minutes of 30 mmHg had a 6 week survival rate of 0/6 (0%); rats subjected to 60 minutes of 30 mmHg had a 6 week survival rate of 0/6 (0%) ($p < 0.01$ by

Fisher Exact Test). There were no significant differences of blood gas levels among these 4 groups. There is a critical period between 30 minutes and 35 minutes of hemorrhagic shock in which survival plummets from 67 % down to 16.7%. We decided to use this protocol to investigate whether prophylactic remote ischemic preconditioning could improve long term survival and the function and structure of the vital organs (heart, brain, lung, kidney and liver) after experimental hemorrhagic shock in the setting of mean blood pressure 30 mmHg maintained for 30 minutes. In the future we would like to determine if periods of 10-20 minutes of hypotension in this model are associated with better or even completely reversible shock and determine exactly when irreversible shock (inability to recover after restoration of shed blood) begins to occur.

From 6 Jun 2016 to 30 Aug 2017, after 9 months of above mentioned pilot studies, we formally started to instrument rats and perform studies for Specific Aim 1. Fixed-pressure hemorrhagic shock was induced in Sprague-Dawley rats (mixed gender). The rats were anesthetized with intraperitoneal ketamine and xylazine, and were heparinized with 500 U/kg heparin. Hemorrhagic shock was induced by removing blood via the left carotid artery catheter to attain a mean blood pressure of 30 mm Hg. Mean blood pressure was maintained at 30 mm Hg for 30 minutes and then the collected blood was returned over the next 30 minutes. At 60 minutes after closely monitoring the animals and returning the shed blood, the catheters were removed from the blood vessels and the rats were allowed to recover from anesthesia, and then allowed to survive for 6 weeks. The impact of remote ischemic preconditioning on both short-term and long-term survival (6 weeks) and long-term organ structure and function were assessed.) Rats subjected to preconditioning had a 6 week survival rate of 8 out of 19 (42.1 %), and rats subjected sham preconditioning had a 6 week survival rate of 3 out of 16 (18.8 %) (Figure 2). There is therefore a strong trend favoring improved survival in the preconditioned group. We are planning on continuing this study into the first few months of year two to achieve about 44 rats in each group which was our original estimate of number of animals needed. Measurement of lactate at 60 minutes after restoration of shed blood was significantly lower in the remote ischemic preconditioning group compared to the control group (Figure 3). There were no differences in the levels of blood gases, sodium, potassium and chloride in preconditioning group vs control group. Unexpectedly, reticulocyte count (%) was significantly increased in preconditioning group (2.99 ± 0.17) compared to control group (2.5 ± 0.13 ; $p=0.044$) (Figure 3). This indicated that there was an increased compensatory production of reticulocytes to replace the lost red blood cells. The mean blood pressure was comparable between the 2 groups during the baseline, shock and resuscitation phases. The left ventricular diastolic and systolic diameter, and fractional shortening were similar between the 2 groups at baseline, end of 30 mmHg hypotension, and end of 1 hour after shed blood reinfusion. This is an important finding as it suggests that the development of irreversible shock, whereby blood pressure does not recover after re-infusion of the lost blood volume, is not due to stunned myocardium.

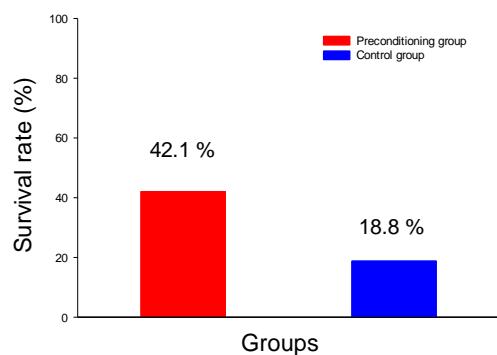


Figure 2: Comparison of survival rate between the 2 groups at 6 weeks after hemorrhagic shock. Rats subjected to preconditioning had a survival rate of 8 out of 19 (42.1 %), and rats subjected to sham preconditioning had a survival rate of 3 out of 16 (18.8 %). There is therefore a strong trend favoring improved survival in the preconditioned group.

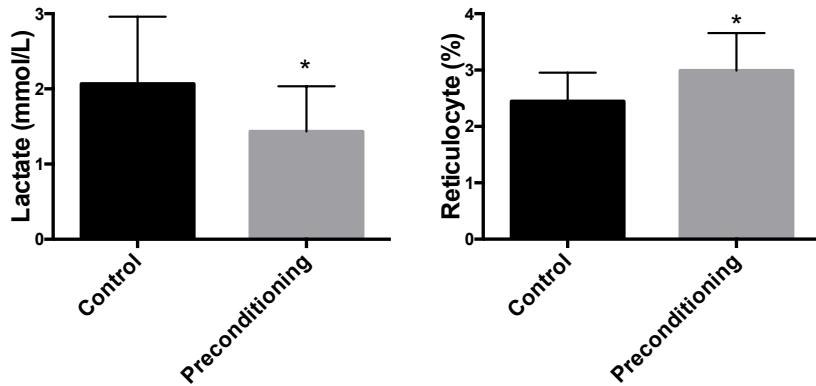


Figure 3: Biochemical results. Lactate was significantly lower in the remote ischemic preconditioning group (1.43 ± 0.14 mmol/L) compared to the control group (2.07 ± 0.24 mmol/L; $p=0.024$). Reticulocyte count (%) was significantly increased in preconditioning group ($2.99 \pm 0.17\%$) compared to control group ($2.5 \pm 0.13\%$; $p=0.044$).

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project.

“Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The present studies have resulted in 3 abstracts that are accepted for the American Heart Association Scientific Sessions 2017, which will be held in Anaheim, CA, November 11-15, 2017

1. Wangde Dai, Jianru Shi, Sharon Hale, Marie Csete, Juan Carreno, and Robert A. Kloner. Protective effect of isoflurane in an experimental model of hemorrhagic shock.
2. Wangde Dai, Jianru Shi, Juan Carreno, Sharon Hale, and Robert A. Kloner . Relationship between survival and duration of hypotension in rat fixed pressure hemorrhagic shock model: the first critical 30 minutes.
3. Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner. Blood-based biomarkers as early predictors of long term outcome in an experimental model of hemorrhagic shock

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

During next reporting period, we will continue to complete the studies of prophylactic remote ischemic preconditioning in Specific Aim 1.

In order to characterize this fixed pressure hemorrhagic shock model under K/X anesthesia, we would like to add some additional groups to the dose finding study, such as 10, 15, or 20 min of hypotension (30 mmHg; n= 6 in each group), to figure out the hypotension duration that would result in 100% survival rate (reversible hypotension) versus when irreversible hypotension (lack of survival after restoration of blood) begins to occur.

In our pilot study, we have demonstrated that isoflurane may have protective effects in the setting of hemorrhagic shock compared to K/X, but the n number (n=6 in each group) is small. We would like to study more isoflurane vs K/X animals at 60 minutes in the hypotension level of 30 mmHg (n= 6 in each group), to verify these findings. We would also like to consider future investigations into a host of other anesthetic agents that might be protective; perhaps as part of future grant proposals.

After completing the studies in Specific Aim 1, we will focus on the studies in Specific Aim 2 to test the hypothesis that prophylactic administration of a mitochondrial protective agent improves long term survival and the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. While we initially proposed studying the drug SS-31, the company that makes this drug suggests an alternative mitochondrial protective drug that is as or stronger than the initial agent that we suggested. We are planning on starting this study in the first half of year 2, but because of our unexpected cardio-protective findings with isoflurane, which we think is a very important observation, we are planning on studying an additional 6 rats in an isoflurane versus ketamine/xylazine first as well as finishing up the time course study adding a 10, 15 minute and/or 20 minute group first.

We are requesting two sham rats not exposed to hypotension as controls for our histology analysis.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Results from our dose finding study using isoflurane suggest that this anesthetic agent may have protective effects on the whole body, as it was shown to have protective effects in previous studies of experimental myocardial infarction. Isoflurane markedly improved survival in our model of hemorrhagic shock, compared to standard ketamine and xylazine. Isoflurane showed better recovery of blood pressure early after transfusion/ resuscitation, as well as long term survival without end-organ infarction. This finding was unexpected but may have very important clinical implications for wounded soldiers suffering blood loss. Whether this beneficial effect is unique to only isoflurane or occurs with other anesthetics needs to be further investigated. In the future we would like to test a series of various anesthetic agents to determine whether others appear more protective; perhaps as part of a new grant. These findings could form the basis of important clinical trials.

Data so far suggest that remote ischemic preconditioning improves long term survival, reduces the production of lactate and stimulates formation of reticulocytes. If this therapy continues to show protection as we move forward, then it has a major clinical implication for the military. It suggests that a cost effective maneuver of inflating and deflating a blood pressure cuff on the arm of a combat soldier, prior to going into battle, could provide protection from dying from hemorrhagic shock. If the findings remain positive then this study could form the basis of an important clinical trial, testing remote ischemic conditioning in soldiers.

Our dose finding study suggested that there is a critical period of time at which hemorrhagic shock at a level of 30 mmHg becomes irreversible – that is restoration of the shed blood still does not save the subject. It appears that 30-35 minutes of hypotension is a critical period. Future studies may better define this and show that 15-20 minutes or earlier is always reversible (meaning that restoration of shed blood always results in survival of the subject).

In order to help improve management it would be useful to have early biomarkers to aid in the prediction of death versus survival in the setting of hemorrhagic shock. As part of a dose and time course finding study we examined early blood-based biomarkers in 37 rats undergoing hemorrhagic shock. Sprague Dawley rats were anesthetized with ketamine/xylazine or Isoflurane, intubated and ventilated with room air. Rats were heparinized and subjected to blood withdrawal hypotension at a fixed level of 30 or 40mmHg for 30 minutes, 35 minutes, 45 minutes, or 60 minutes and then the shed blood was reinfused. Arterial blood samples were collected at 1 to 3 hours after returning shed blood and then the rats were allowed to survive for 6 weeks. Rats that died (n=25) showed early significantly elevated potassium levels compared to rats that survived (n=12; death: 6.56 ± 0.22 mmol/L; survival: 4.98 ± 0.23 mmol/L; p=0.00002); had higher chloride levels (death: 113.4 ± 0.99 mmol/L; survival: 109.1 ± 0.75 mmol/L; p=0.0014) and had higher sodium levels (death: 138.52 ± 0.78 mmol/L; survival: 135.77 ± 0.72 mmol/L; p=0.014). The pH (death: 7.27 ± 0.03 ; survival: 7.41 ± 0.02 ; p=0.0002) and base excess (death: -8.14 ± 1.17 mmol/L; survival: -0.04 ± 0.96 mmol/L; p=0.000006) levels were significantly lower in rats that died versus survivors. There were no differences in PO₂, PCO₂, hematocrit, hemoglobin, glucose and lactate between rats that died versus survived. The results demonstrated that early blood based biomarkers may help predict the long term prognosis in hemorrhagic shock. Within the first few hours after blood resuscitation, high potassium, chloride and sodium levels coupled with low pH and low base excess lead to a negative prognosis. (Also see AHA abstract at the end of the report).

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

It could be that if isoflurane and or preconditioning work in this model that it might work in other types of shock that include not only hypotension but tissue trauma, septic shock, and other forms of ischemia/reperfusion injury to organs.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Because there was a significant difference in the survival rate under anesthesia with isoflurane versus ketamine/xylazine in rats that were subjected to hemorrhagic shock, the finding suggests that the type of anesthesia used during surgery in hemorrhagic shock situations may need to be re-evaluated. Isoflurane appears to be more protective than the ketamine/xylazine.

In addition we are observing a trend for an improved survival with remote ischemic preconditioning. The results are suggestive of a benefit, and if this study pans out, it would be relatively easy to translate this to a clinical study whereby soldiers going into battle precondition themselves beforehand using a blood pressure cuff and inflation over the brachial artery with 5 minutes of inflation and 5 minutes of deflation repeated 3-4 times.

Could change behavior in battle field depending on results of the remote ischemia preconditioning study.

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We are requesting some changes based upon our findings from year 1. We would like to further explore the dose finding study and see if 10, 15 minutes and/or 20 minutes of hypotension at 30 mmHg are associated with less irreversible shock in rats (n=6 in each group). We think it is important to determine the cut off for reversible shock (when blood can be returned and the animal survives) , versus irreversible shock (when blood is returned but the animal dies, anyway.) So far it appears that a 30-35 mmHg time point may be the point of no return.

We would like to increase n values in the isoflurane study to verify the reproducibility of the protective effects of isoflurane versus ketamine/xylazine in hemorrhagic shock by adding another 6 rats to each group.

The mitochondrial targeted agent that we are evaluating Elamipretide or SS 31 may no longer be available from the company that was originally going to supply it. They are planning on supplying us a mitochondrial agent that is equally or even more powerful than this agent.

We would like to add two sham rats to serve as normal controls for histology of lungs, brains.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We did have a delay in finding the correct dose and timing of hypotension to allow a death rate that was not so severe but not so low as to allow us to determine if remote ischemic conditioning would be of benefit on survival. During this dose finding study we made some unexpected observations and think that the findings with isoflurane may be even more important than the original therapies we suggested. We are therefore requesting a chance to increase the numbers in the isoflurane study to about 6 in each group, and increase the dose finding study by adding 2-3 more time points to determine the exact time course of when death occurs.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

Adding more rats to the isoflurane study (n=about 12).
Adding additional animals to time course study (n = about 12-18).
We will also be requesting 2 sham animals to serve as control for histologic analysis.
These additions will be submitted shortly to our IACUC and then your ACURO.
We do not think that these additions of small numbers of animals will increase the overall cost.

Significant changes in use of biohazards and/or select agents

No change in biohazards. We will be using an alternative mitochondrial protective agent, rather than SS-31 as described above.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

There are 3 abstracts that will be in press in the journal Circulation.

1. Wangde Dai, Jianru Shi, Sharon Hale, Marie Csete, Juan Carreno, and Robert A. Kloner. Protective effect of isoflurane in an experimental model of hemorrhagic shock.
2. Wangde Dai, Jianru Shi, Juan Carreno, Sharon Hale, and Robert A. Kloner. Relationship between survival and duration of hypotension in rat fixed pressure hemorrhagic shock model: the first critical 30 minutes.
3. Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner. Blood-based biomarkers as early predictors of long term outcome in an experimental model of hemorrhagic shock

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

None yet

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

There are 3 abstracts that have been accepted to present in the American Heart Association Scientific Sessions 2017, which will be held in Anaheim, CA, November 11-15, 2017.

1. Wangde Dai, Jianru Shi, Sharon Hale, Marie Csete, Juan Carreno, and Robert A. Kloner. Protective effect of isoflurane in an experimental model of hemorrhagic shock.
2. Wangde Dai, Jianru Shi, Juan Carreno, Sharon Hale, and Robert A. Kloner . Relationship between survival and duration of hypotension in rat fixed pressure hemorrhagic shock model: the first critical 30 minutes.
3. Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner. Blood-based biomarkers as early predictors of long term outcome in an experimental model of hemorrhagic shock

(The 3 abstracts are attached in Section 9 - APPENDICES)

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

None.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Not applicable

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Not applicable

- **Other Products**

Identify any other reportable outcomes that were developed under this project.

Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *biospecimen collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

OTHER: Reports at scientific sessions. 3 abstracts will be presented in the resuscitation science symposium section of the upcoming American Heart Scientific Sessions, in Anaheim, CA, Nov 2017

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change.”

Example:

Name: Mary Smith

Project Role: Graduate Student

Researcher Identifier (e.g. ORCID ID): 1234567

Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding

support is provided from other than this award).

1. Name:	Robert A. Kloner
Project Role:	Principle investigator
Nearest person month worked:	6 months (50% per year)
Contribution to Project:	Dr. Kloner has performed work in the area of study design, data collection and analysis, report and manuscript writing.
2. Name:	Wangde Dai
Project Role:	Senior investigator
Nearest person month worked:	9.6 months (80% per year)
Contribution to Project:	Dr. Dai has performed work in the animal studies, data collection and analysis, report and manuscript writing.
3. Name:	Jianru Shi
Project Role:	Senior investigator
Nearest person month worked:	5.4 months (45% per year)
Contribution to Project:	Dr. Shi has performed work in the area of blood and tissue sampling, data collection and analysis, report and manuscript writing.
4. Name:	Sharon Hale
Project Role:	Senior investigator
Nearest person month worked:	1 month (8.3% per year)
Contribution to Project:	Mrs Hale has performed work in the area of study design, data collection and analysis, report and manuscript writing.
5. Name:	Juan Carreno
Project Role:	Senior investigator
Nearest person month worked:	0.6 month (5% per year)
Contribution to Project:	Dr. Carreno has performed work in the area of animal handling and caring, data collection and analysis, report and manuscript writing.
6. Name:	Jesus Chavez
Project Role:	Histology technician
Nearest person month worked:	0 month (0% per year) He will start to work on this project next year.
Contribution to Project:	Mr. Chavez has performed work in the area of tissue collection, processing, sectioning, and various staining.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

None.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is

acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Below please find the 3 accepted abstracts for presentation at the American Heart Association Meetings, scheduled for Nov 2017.

Protective effect of isoflurane in an experimental model of hemorrhagic shock

Wangde Dai, Jianru Shi, Sharon Hale, Marie Csete, Juan Carreno, and Robert A. Kloner

HMRI Cardiovascular Research Institute, Huntington Medical Research Institutes, 10 Pico Street, Pasadena, CA 91105, and Division of Cardiovascular Medicine of the Keck School of Medicine, University of Southern California, Los Angeles, California 90017-2395.

Background: Hemorrhagic shock is a leading cause of preventable death in young adults, both military and civilian. The volatile anesthetic isoflurane can protect (precondition) against cardiac reperfusion injury but has not been widely studied as a preconditioning agent in the setting of hemorrhagic shock (and restoration of blood volume).

Methods: Sprague Dawley rats (both genders) were anesthetized with either intraperitoneal ketamine and xylazine (K/X, 90 mg/kg and 10 mg/kg; n=6), or with isoflurane (5% isoflurane induction and 2% maintenance in room air; n=6). Rats were intubated and ventilated with room air, heparinized, and hemorrhagic shock induced by withdrawing blood from the left carotid artery to a fixed mean blood pressure of 30 mm Hg for one hour, followed by 30 min of resuscitation with shed blood. Rats remained anesthetized for one hour during which hemodynamics were monitored (femoral artery catheter); and then allowed to survive for 6 weeks.

Results: The survival rate at 6 weeks in rats receiving K/X was 0/6 (0%); the survival rate in rats receiving isoflurane was 5/6 (83%; p<0.05 by Fisher's exact test). All deaths occurred within 24 hours of the shock study; rats surviving past 24 hours survived for 6 weeks. Recovery of blood pressure during the resuscitation phase was significantly improved in the isoflurane group compared to the K/X group. (Figure below). At 6 weeks, all rats in the isoflurane group were neurologically intact, and tetrazolium chloride staining showed no evidence of myocardial, brain or kidney infarction.

Conclusions: These results suggest that isoflurane may have protective effects in the setting of hemorrhagic shock, marked by improved recovery of blood pressure early after transfusion/resuscitation, as well as long term survival without end-organ infarction.

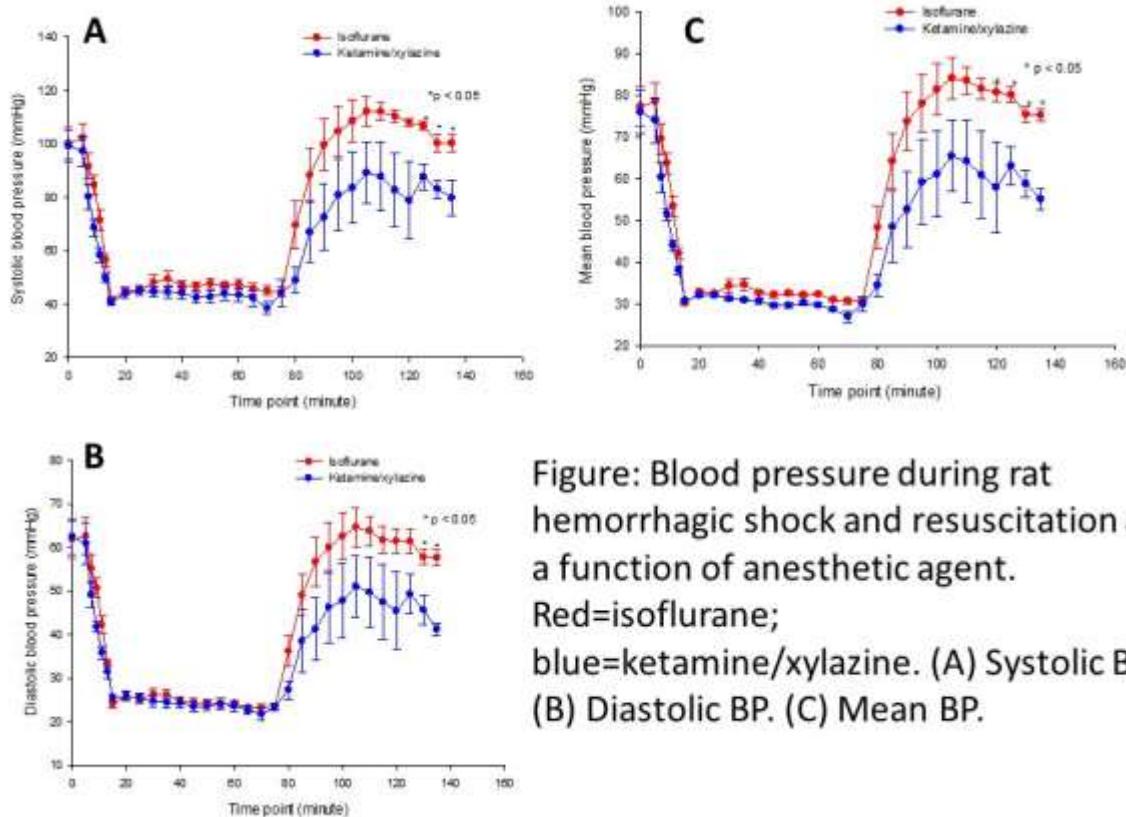


Figure: Blood pressure during rat hemorrhagic shock and resuscitation as a function of anesthetic agent. Red=isoflurane; blue=ketamine/xylazine. (A) Systolic BP. (B) Diastolic BP. (C) Mean BP.

Relationship between survival and duration of hypotension in rat fixed pressure hemorrhagic shock model: the first critical 30 minutes

Wangde Dai, Jianru Shi, Juan Carreno, Sharon Hale, and Robert A. Kloner

HMRI Cardiovascular Research Institute, Huntington Medical Research Institutes, 10 Pico Street, Pasadena, CA 91105, and Division of Cardiovascular Medicine of the Keck School of Medicine, University of Southern California, Los Angeles, California 90017-2395.

Background: Hemorrhagic shock is a leading killer of soldiers in the battle field. In order to develop a reliable experimental hemorrhagic model for developing new therapeutic strategies for preventing hemorrhagic-shock- induced death, we determined how long hypotension could be tolerated before death was inevitable.

Methods: Sprague Dawley rats (mixed gender) were anesthetized with intraperitoneal ketamine and xylazine (90mg/kg and 10mg/kg), intubated and ventilated with room air. Rats were heparinized and subjected to blood withdrawal via catheter implanted in the left carotid artery in order to establish hypotension at a level of 30 mmHg. Rats were subjected to this level of hypotension for 30 minutes, 45 minutes, or 60 minutes and then the shed blood was reinfused within 30 min (n=6 in each group). The rats were monitored under anesthesia for one hour and then allowed to survive for 6 weeks.

Results: Rats exposed to 30 minutes of 30 mmHg had a 6 week survival rate of 4/6 (66.7%); rats subjected to 45 minutes of 30 mmHg had a 6 week survival rate of 0/6 (0%); rats subjected to 60 minutes of 30 mmHg had a 6 week survival rate of 0/6 (0%) ($p=0.01$ by Fisher Exact Test). Rats that died did so during the first 24 hours; those surviving for 24 hours survived for 6 weeks. At 6 weeks none of the survivors showed gross neurologic deficits. At 5 min after starting of shed blood reinfusion, the mean blood pressure was significantly higher in 30 min (52 ± 2 mmHg) and 45 min (48 ± 3 mmHg) groups compared to 60 min (34 ± 3 mmHg; $p=0.001$) group. In general, rats that died demonstrated a fall in blood pressure within an hour of restoring blood volume, suggesting that there may have been a vasodilation of blood vessels and/or a vascular leak problem.

Conclusions: There is a critical period between 30 minutes and 45 minutes of hemorrhagic shock in which survival plummets from 67 % down to zero; attempts should be made to restore blood volume within at least 30 minutes of hemorrhagic shock in order to improve survival.

Blood-based biomarkers as early predictors of long term outcome in an experimental model of hemorrhagic shock

Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner

Background: Hemorrhagic shock is the leading cause of death on the battlefield. To help improve management it would be useful to have early biomarkers to aid in the prediction of death versus survival in the setting of hemorrhagic shock. As part of a dose and time course finding study we examined early blood-based biomarkers in 37 rats undergoing hemorrhagic shock. Our purpose was to identify early biomarkers that best predicted long term survival versus death.

Methods and results: Sprague Dawley rats were anesthetized with ketamine/xylazine or Isoflurane, intubated and ventilated with room air. Rats were heparinized and subjected to blood withdrawal hypotension at a fixed level of 30 or 40mmHg for 30 minutes, 35 minutes, 45 minutes, or 60 minutes and then the shed blood was reinfused. Arterial blood samples were collected at 1 to 3 hours after returning shed blood and then the rats were allowed to survive for 6 weeks. Rats that died ($n=25$) showed early significantly elevated potassium levels compared to rats that survived ($n=12$; death: 6.56 ± 0.22 mmol/L; survival: 4.98 ± 0.23 mmol/L; $p=0.00002$); had higher chloride levels (death: 113.4 ± 0.99 mmol/L; survival: 109.1 ± 0.75 mmol/L; $p=0.0014$) and had higher sodium levels (death: 138.52 ± 0.78 mmol/L; survival: 135.77 ± 0.72 mmol/L; $p=0.014$). The pH (death: 7.27 ± 0.03 ; survival: 7.41 ± 0.02 ; $p=0.0002$) and base excess (death: -8.14 ± 1.17 mmol/L; survival: -0.04 ± 0.96 mmol/L; $p=0.000006$) levels were significantly lower in rats that died versus survivors (Figure). There were no differences in PO₂, PCO₂, hematocrit, hemoglobin, glucose and lactate between rats that died versus survived.

Conclusions: Early blood based biomarkers may help predict the long term prognosis in hemorrhagic shock. Within the first few hours after blood resuscitation, high potassium, chloride and sodium levels coupled with low pH and low base excess lead to a negative prognosis.

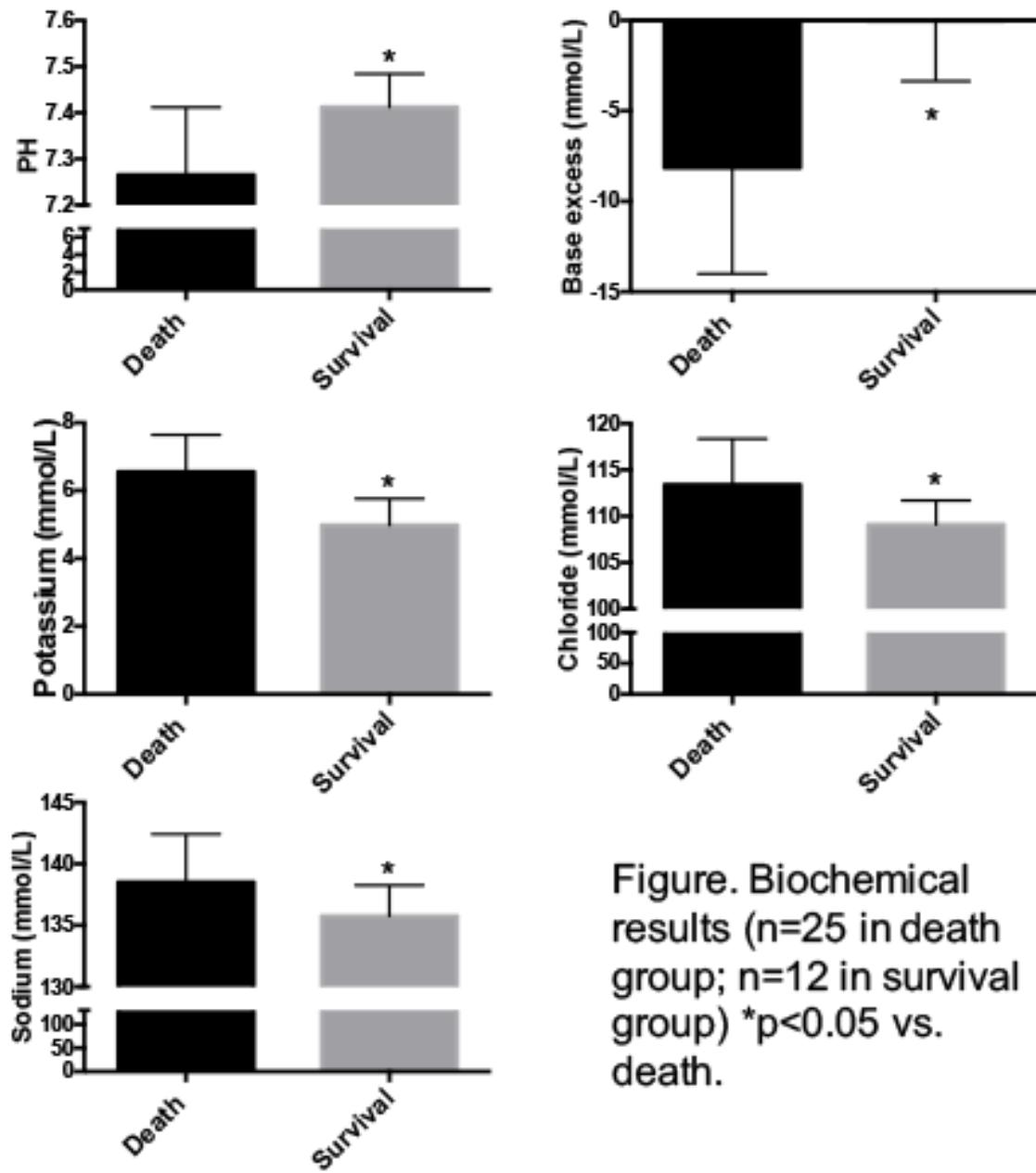


Figure. Biochemical results (n=25 in death group; n=12 in survival group) * $p<0.05$ vs. death.